2061

Recherches préliminaires sur la structure de la partie glucidique des caséino-glycopeptides*

Résumé

Quelques expériences préliminaires concernant le groupement prosthétique glucidique des caséino-glycopeptides sont décrites: l'acide sialique et le galactose occupent une position «externe» par rapport à la galactosamine sans doute liée à la partie peptidique.

La caséine entière de vache privée de sa fraction $\varkappa$ est pratiquement dépourvue de sucres[1]. Ce fait suggère que probablement tous les sucres se trouvent localisés dans la caséine $\varkappa$ qui est la seule fraction glycoprotéique importante de la caséine. Sous l'effet de la présure, à *p*H 6,8, même en absence d'ions Ca, la caséine $\varkappa$ donne naissance à deux fractions: l'une, insoluble, appelée paracaséine $\varkappa$; l'autre, très soluble, appelée «caséino-glycopeptide $\varkappa$».[2] Rappelons que la présure agit sur la seule fraction $\varkappa$ de la caséine entière, qui est ainsi le véritable substrat de l'enzyme[3]. Le caséino-glycopeptide $\varkappa$ contient la majeure partie des sucres de la caséine, à savoir, du galactose, de la galactosamine et de l'acide neuraminique; le caséino-glycopeptide est donc la fraction glucidique la plus importante de la caséine[4]. L'étude de son groupement prosthétique glucidique peut être abordée en travaillant soit avec la caséine soit avec le caséino-glycopeptide: quelques expériences préliminaires ont été effectuées en faisant appel à des méthodes chimiques (action de H_2SO_4 0,1 N; action de l'acide périodique) ou à une méthode enzymatique (action de la neuraminidase).

Composition en sucres des CGP

Le tableau 1 indique la composition en sucres des caséino-glycopeptides (CGP) de vache (provenant de la caséine $\varkappa$), de brebis (provenant de la caséine entière) et de chèvre (provenant de la caséine entière): les rapports moléculaires ont été calculés en admettant que les poids moléculaires des CGP sont de l'ordre de 8000. Les CGP de brebis et de chèvre contiennent un mélange des acides N-acétyl- et N-glycolyl-neuraminiques.

Tableau 1. Composition en sucres de trois caséino-glycopeptides en % de substance sèche; r = rapport moléculaire calculé en fixant le poids moléculaire des CGP égal à 8000

Caséino-glycopeptide	Galactosamine* %	r	Galactose** %	r	Acide sialique*** %	r
CGP $\varkappa$ de vache	6,5	2,9	6,0	2,7	10,2	2,8
CGP de brebis	2,4	1,0	2,1	0,9	1,0	0,3
CGP de chèvre	3,4	1,5	3,6	1,6	2,8	0,7

* Selon RONDLE et MORGAN[5].
** Selon SCHULTZE et coll.[6]
*** Exprimé en acide N-acétylneuraminique (PM 309), dosage selon WARREN après hydrolyse acide[7].

Action de la neuraminidase sur la caséine $\varkappa$ et les CGP

Lorsque l'on fait agir la neuraminidase de *V. cholerae*, à *p*H 6 (solution tampon de phosphates 0,03 M) pendant 3 heures à 37°, sur une solution à 1% de caséine $\varkappa$ de vache ou de l'un des trois CGP mentionnés dans le tableau 1, la totalité des acides neuraminiques est libérée: l'acide neuraminique occupe donc une position terminale.

Action du périodate de K sur la caséine $\varkappa$

A une solution à 1% de caséine $\varkappa$ de vache dans une solution tampon de phosphates 0,05 M; *p*H 7; contenant

* Reçu le 10 octobre 1964.

[1] C. ALAIS, Thèse Sci., Paris 1962; C. ALAIS et P. JOLLÈS, *Lait 44* (1964) 138.
[2] P. JOLLÈS, C. ALAIS et J. JOLLÈS, *Biochim. Biophys. Acta 51* (1961) 309.
[3] D. F. WAUGH et P. H. VON HIPPEL, *J. Amer. Chem. Soc. 78* (1956) 4576.
[4] C. ALAIS et P. JOLLÈS, *Biochim. Biophys. Acta 51* (1961) 315.
[5] C. J. RONDLE et W. T. J. MORGAN, *Biochem. J. 61* (1955) 586.
[6] H. E. SCHULTZE, R. SCHMIEDTBERGER et R. HAUPT, *Biochem. Z 329* (1958) 490.
[7] L. WARREN, *J. Biol. Chem. 234* (1959) 1971.

du KCl (0,15 M), on ajoute du périodate de K (35 mg KIO_4 pour 100 mg de caséine $\varkappa$); 80% de l'acide sialique sont détruits en 30 minutes à température ordinaire; par contre, les teneurs en galactose et en galactosamine demeurent constantes.

Action de la neuraminidase suivie de celle du périodate de K sur la caséine $\varkappa$

On fait agir cette fois le périodate de K sur une solution de caséine $\varkappa$ préalablement incubée avec de la neuraminidase, donc privée de l'acide sialique terminal. Après 30 minutes à température ordinaire, 40% de galactose ont disparu: par contre, la teneur en galactosamine demeure constante. Il semble donc que ce soit le galactose qui après le départ de l'acide sialique occupe une position «externe».

Action de l'acide sulfurique 0,1 N sur les CGP

Les CGP de vache ($\varkappa$), de brebis et de chèvre sont hydrolysés pendant 6 heures à 100° avec H_2SO_4 0,1 N[8,9]. L'hydrolysat est dessalifié puis chromatographié sur papier Whatman n° 1 dans le solvant *n*-butanol-acide acétique-eau (4 : 1 : 5, v/v). Deux substances ont pu être identifiées avec le réactif à l'oxalate d'aniline: le galactose libre (très abondant) et une substance peu abondante de $R_{GlcNH_2} = 0{,}82$. Il n'a pas été possible de caractériser de la galactosamine libre.

D'autre part, la substance de $R_f = 0$ a été éluée du papier puis analysée. Elle contient de nombreux acides aminés et de la galactosamine (liée aux acides aminés); par contre, il n'a pas été possible de déceler la présence de galactose. Ces expériences montrent que c'est le galactose qui, après la destruction de l'acide sialique, occupe une position «externe»; par contre, c'est la galactosamine qui semble être directement liée à la partie peptidique.

Conclusion

La labilité particulière des acides sialiques et du galactose permet de placer ces composés en position «externe» par rapport à la galactosamine plus «interne». Ces trois glucides se trouvent en proportions équimoléculaires dans le CGP de vache. Dans les deux autres CGP, la galactosamine et le galactose se trouvent en proportions équimoléculaires, par contre, les résultats du dosage des acides sialiques indiquent qu'il y a 2 à 3 fois moins de résidus de ces composants; mais il s'agit de substances particulièrement labiles: le dosage, dans des matières qui en contiennent très peu, manque de précision.

Des recherches ultérieures devront déterminer si le groupement prosthétique du CGP de vache est formé par trois enchaînements identiques:

$$\text{Acides aminés} \longrightarrow \text{Gal}_{NH_2} \longrightarrow \text{Gal}$$
$$\longrightarrow \text{Acide N-acétylneuraminique}$$

ou par une seule chaîne contenant les 9 résidus glucidiques groupés 3 par 3. La nature de la liaison entre la partie peptidique et la galactosamine qui, d'après nos expériences, ne semble pas être du type ester, est également en cours d'étude.

En ce qui concerne les CGP de brebis et de chèvre, de nouvelles déterminations des composants glucidiques seront faites afin de préciser les rapports moléculaires.

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[8] J. Montreuil et A. Chosson, *C. R. Acad. Sci. 255* (1962) 3071.
[9] J. Montreuil, G. Spik et A. Chosson, *C. R. Acad. Sci. 255* (1962) 3493.

Bernardi *et al.*, (1963), and Bernardi and Griffé (1964). Both crude and highly purified enzyme preparations were used.

Papain was twice-crystallized Worthington papain; 0.2 ml of the suspension was dissolved in 3 ml of distilled water; 0.02 or 0.05 ml of this solution was added to 30 ml DNA solutions (50–100 μg/ml) in acetate buffer, *p*H 5.4, $\mu = 0.15$. The final enzyme concentrations were about 1 or 2.5 μg/ml, respectively.

Chymotrypsin was three-times-crystallized Worthington α-chymotrypsin. A 0.1% aqueous solution (0.03 ml) was added to 30 ml DNA solutions (50–100 μg/ml) in phosphate buffer, $\mu = 0.1$, *p*H 6.8. The final enzyme concentration was therefore about 1 μg/ml.

Pancreatic DNAase was once-crystallized Worthington DNAase (lot 932). The enzyme was dissolved in acetate buffer, $\mu = 0.2$, *p*H 5.5, containing 0.02 M $MgCl_2$, at a concentration of about 0.5 mg/ml. Aliquots of 0.05–0.01 ml of freshly prepared solution were added to 30 ml of DNA (90–140 μg/ml) in the same solvent. The final enzyme concentration was of the order of 0.1 μg/ml.

Methods

Nitrogen was determined by the micro-Kjeldahl procedure, and phosphorus by the method of Martin and Doty (1949). Ultraviolet absorption, sedimentation and light-scattering measurements were performed as already described (Bernardi *et al.*, 1961).

In the more recent light-scattering work, a new clarification technique (Bernardi, 1964), also successfully used in a low-angle light-scattering investigation of DNA solutions (Froelich *et al.*, 1963), was employed. Couette viscometry was performed, as in previous work (Bernardi *et al.*, 1961), using an instrument built by Dr. G. Scheibling of this laboratory.

Enzymatic digestion was performed, at least in duplicate, directly in the light-scattering cell at room temperature (20–22°). Unless otherwise stated, 0.01 or 0.02 ml of crude or commercial DNAase solution (1 mg/ml) was added to 30 ml of DNA (50–150 μg/ml), the final enzyme concentration being about 0.3 μg/ml. Much lower concentrations were used for the pure enzyme preparations; bovine serum albumin was added to these preparations in order to stabilize them. Routinely, readings were taken at only six angles between 30° and 90°. When plotting Kc/R versus $\sin^2(\theta/2)$, the points belonging to the four lower angles (often all the points) were on a straight line and extrapolation to zero angle was performed linearly to obtain $1/M_w$.

Acid-soluble oligonucleotides were determined as described in the following paper (Bernardi and Griffé, 1964). For heat denaturation of intact and partially digested DNA, in a typical experiment, a large DNA sample (for instance 500 ml of a 0.5 mg/ml solution) was digested in standard acetate buffer at 37° and aliquots were removed at different times. Digestion was stopped by cooling these in an ice-bath and shaking them with chloroform-isoamyl alcohol. An intact sample of the starting DNA solution was treated in the same way. All samples were dialyzed against Na acetate, $\mu = 0.2$, *p*H 8.0, and diluted to give a DNA concentration of 10 μg/ml. For heat denaturation the samples were put in a boiling-water bath for different times, then rapidly cooled in an ice bath, shaken with chloroform-isoamyl alcohol, dialyzed back to standard acetate buffer, shaken once more with chloroform-isoamyl alcohol, and used for the light-scattering experiments. This rather complicated procedure was the only one capable of giving solutions which were perfectly stable for at least 24 hours at room temperature, as judged from the light-scattering envelopes. The same solutions in neutral or slightly alkaline buffers invariably showed a rapid aggregation. The same solutions were used for the determinations of optical density, phosphorus, and acid-soluble-oligonucleotide content.

Treatment of Kinetic Data

The statistical theory of random degradation of linear polymers of any initial molecular-weight distribution predicts that $1/M_n$ is a linear function of time t when the number of chain scissions is proportional to (Mark and Tobolsky, 1950). It has been shown by Charlesby (1954) that any initial molecular-weight distribution yields the most probable one after at small extent of degradation (three to eight chain fractures per molecule). For the most probable molecular-weight distribution, $M_w = 2M_n$, and $1/M_w$ will also be a linear function of t.

Schumaker *et al.* (1956) have given a general theory for the degradation of multistranded polymers of any initial distribution of molecular weights. They have shown that for the initial stage of degradation the following equation holds:

$$\log (1 - R) = n \log p + \text{constant} \qquad (1)$$

Here, $R = M_t/M_0$ (M_t and M_0 are the molecular weights at time t and at time 0, respectively), n is the apparent number of strands, and p is the probability that any given bond be broken at time t. Equation (1) shows that a plot of $\log (1 - R)$ versus $\log p$ will have a slope equal to n. A more convenient plot is obtained by replacing, as proposed by Cavalieri and Rosenberg (1961), $(1 - R)$ by $(1 - R)/R$ in equation (1). Then, assuming that $p = kt$ (k being a proportionality constant), equation (1) becomes:

$$\log (1 - R)/R = n \log t + \text{constant} \qquad (2)$$

The advantage of this representation is that $\log (1 - R)/R$ is a linear function of $\log t$ over a wider range of R values. For the particular case where $n = 1$, equation (2) becomes:

$$\frac{1}{M_t} - \frac{1}{M_0} = kt \qquad (3)$$

that is, the reciprocal of molecular weight is a linear function of t, as indicated.

Two more treatments of the kinetic data obtained during the degradation of DNA have been proposed by Thomas (1956) and Applequist (1961), respectively. Both methods require data obtained during a rather extensive degradation, down to values of R of the order of 0.1 or lower; in the former method titration data are also required. In the present work the treatment of Schumaker *et al.* (1956) was preferred because it utilizes data obtained during the very early stage of the enzymic degradation, a period where the assumption $p = kt$ is justified, and interference by other enzymes or inhibition phenomena are less likely to occur. These advantages were, of course, especially important when titration data and pure enzyme preparations were not yet available.

Results

Degradation of Native DNA with Pancreatic DNAase. —These experiments were performed mainly in order to check our technique and to compare our results with those of Thomas (1956) and Schumaker *et al.* (1956). The data obtained (Figs. 1 and 2; Table I) are in agreement with those already published by the above-

TABLE I
DEGRADATION OF NATIVE DNA BY PANCREATIC DNAASE[a]

DNA Sample	Source	Preparation Method	DNA Concn (μg/ml)	M_w 10^{-6}	R_z (A)	n
(1) B 11/2	E	A	96.5	7.5	1540	2.0
(2) B 13/5	E	B	140	6.6	2470	1.7

[a] See footnotes to Table II.

TABLE II
DEGRADATION OF NATIVE DNA BY ACID DNAASE PREPARATIONS[a]

DNA Sample	Source[b]	Preparation Method[c]	DNA Concn (μg/ml)	M_w 10^{-6}	R_z (A)	Enzyme[d]	n[e]
(1) B6z-N/1	E	A	115	3.8	2080	ET3	1.0
(2) B13/24	E	B	90	7.2	2900	ET3	0.9
(3) B13/30	E	B	86	6.9	2900	ET3	1.0
(4) B13/46	E	B	133	5.5	2570	ERW	1.1
(5) B13/52	E	B	133	5.5	2570	ERP/3	0.9
(6) B6z-N/11	E	A	65	3.8	2080	ET3/3	1.0
(7) B6/15	E	A	75	4.9	2160	(t)HL1	0.9
(8) B3/57	T	A	140	4.6	2120	ERW	1.1
(9) DL1/176	C				2100	(t)HL1	0.9
(10) B6z-N/12	E	A	62.5	3.8	2080	ET3/DFP	1.0
(11) B6/185	E	A		3.4	2330	(t)HL1	0.9
(12) B13/74	E	B			1930	ERW	0.9
(13) B11a/19	E	D	113	4.6	2400	ET2	1.0

[a] All degradations were performed at room temperature (20–22°) in the light-scattering cell, using standard acetate buffer as the solvent, except for samples 7 (acetate buffer, pH 5.4), 11 (0.005 M EDTA, pH 7.2), and 12 (phosphate buffer, pH 6.8, μ = 0.004, containing 10^{-4} M EDTA). [b] E, chicken erythrocytes; T, calf thymus; C, *E. coli*. [c] See Methods. [d] ET3, calf thymus DNAase; ERW, calf spleen DNAase (Worthington); ERP/3, hog spleen pure enzyme; ET3/3, purified calf thymus DNAase of specific activity 50 (see Bernardi and Griffé, 1964); (t)HL1, calf thymus DNAase; ET3/DFP, ET3 treated with diisopropylfluorophosphate. [e] n is the apparent number of strands as calculated from the slope of log $(1 - R)/R$ versus log t (Schumaker *et al.*, 1956).

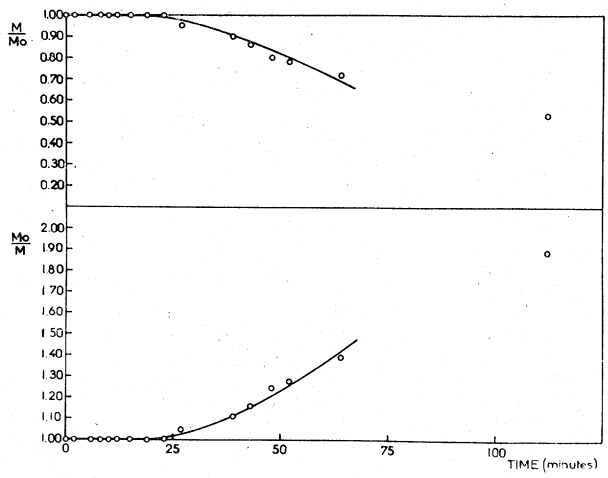

FIG. 1.—Digestion of DNA sample B 11/2 with pancreatic DNAase (see Table I).

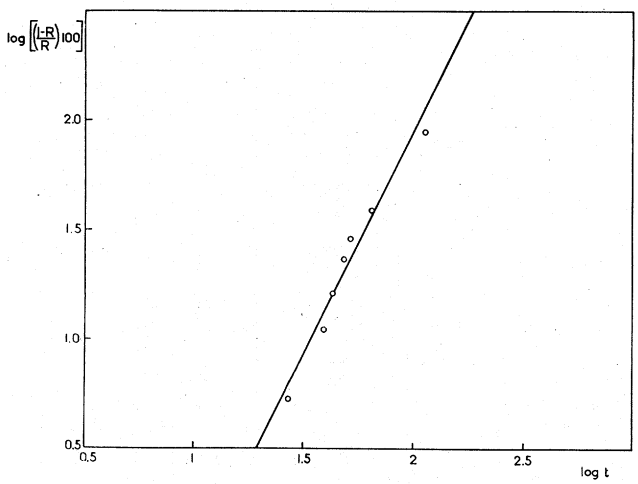

FIG. 2.—Digestion of DNA sample B 11/2 with pancreatic DNAase. Data of Fig. 1 are plotted according to Schumaker *et al.* (1956). See also Table I.

mentioned authors. The molecular weight decrease showed the typical time lag (Fig. 1), and the apparent number of strands was found to be equal to 1.7–2.0 (Fig. 2), as expected. Aggregation phenomena invariably occurred at an early stage, a result in agreement with a similar finding by Thomas (1956).

Degradation of Native DNA with Acid DNAase Preparations.—Many different DNA preparations from calf thymus, chicken erythrocytes, and *E. coli*, obtained using the different preparative procedures described in the experimental section, were digested with acid-DNAase preparations from calf thymus, calf spleen, hog spleen, and chicken erythrocytes.

Some typical results are shown in Table II. They indicate that in all cases the apparent number of strands was found to be equal to 1 ∓ 0.1, in spite of differences in the source, preparation, and concentration of both DNA and enzyme samples used. No differences were found that could be related to the molecular weight and radius of gyration of DNA. Digestions performed with neutral low-molarity buffers as the solvents (Shack, 1957) instead of standard acetate buffer, with enzyme preparations treated with diisopropylfluorophosphate, or with preparations having very different degrees of purity also gave the same result. Figures 3 and 4 show the kinetic data obtained in a typical case.

In all cases, enzymic degradations could be followed

TABLE III
HEAT DENATURATION OF INTACT AND PARTIALLY DIGESTED DNA[a]

DNA Sample	$M_w\ 10^{-6}$		M_{den}/M_{nat}	$\epsilon(P)$[b]		$\epsilon(P)_{den}/\epsilon(P)_{nat}$
	Native	Denatured		Native	Denatured	
B6z/0	3.80	2.20	0.58	6440	6950	1.08
B6z/1	2.35	1.16	0.49	6500	7080	1.09
B6z/2	1.75	0.75	0.43	6450	7280	1.13
B6z/3	1.45	0.54	0.37	6500	7450	1.15

[a] The DNA sample used in this experiment was from chicken erythrocytes. The DNAase preparation used in this digestion was from calf thymus. Heat denaturation was performed as described in the experimental part. Acid-soluble oligonucleotides were not detected in any of the samples, both native and denatured. [b] See Chargaff and Zamenhof (1948).

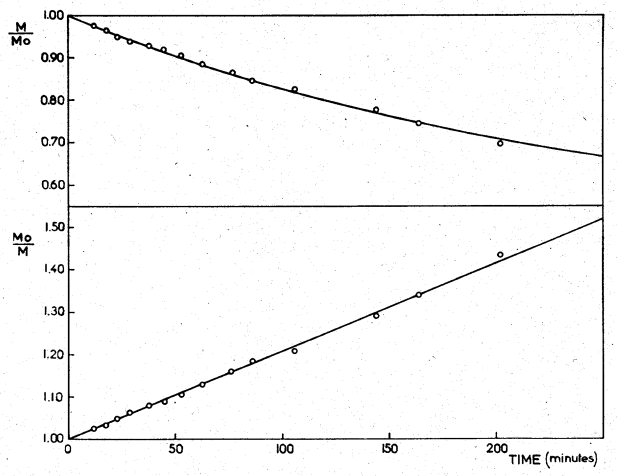

FIG. 3.—Digestion of DNA sample B6zN/1 with acid DNAase (see Table II).

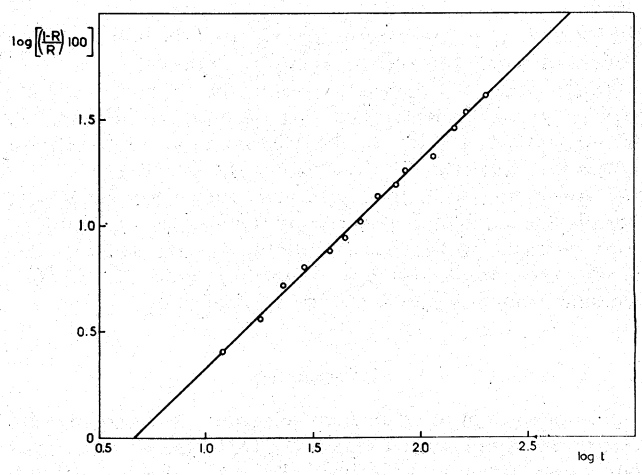

FIG. 4.—Digestion of DNA sample B6zN/1 with acid DNAase. Data of Fig. 3 are plotted according to Schumaker *et al.* (1956). See also Table II.

down to extremely low molecular weights; we did not find aggregation phenomena such as those obtained when using pancreatic DNAase nor enzyme-inhibition effects, of the type found by Cavalieri and Rosenberg (1961) in their digestions of DNA, with very crude acid-DNAase preparations from mouse sarcoma 180.

When the molecular-weight decrease was studied for a long enough time, it was observed that the initial linear increase of $1/M$ with digestion time was followed by a higher-order relationship (Fig. 5); the change took place when a molecular weight of about 1×10^6 was reached. This phenomenon was found without exception in all degradations investigated so far.

Degradation of Single-stranded DNA Samples with Acid-DNAase Preparations.—Samples of ϕ X 174

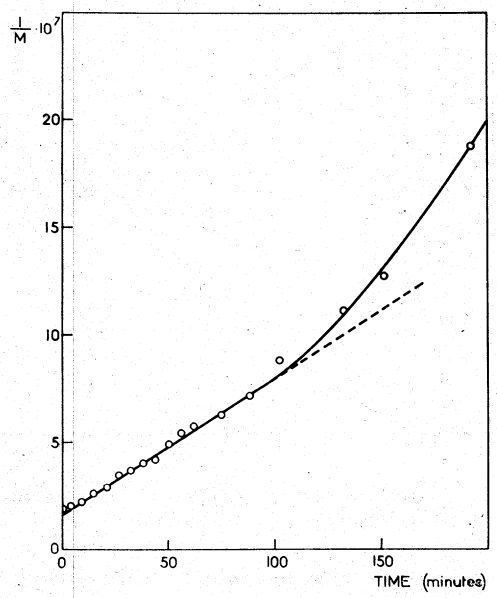

FIG. 5.—Digestion of DNA sample B 13/46 with acid DNAase (see Table II).

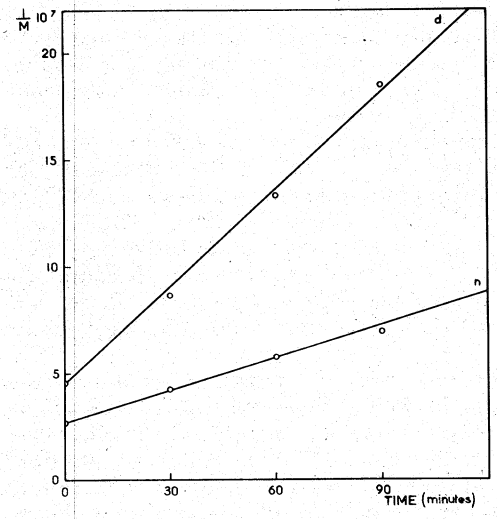

FIG. 6.—Heat denaturation of intact and partially digested DNA (see Table III). A large DNA sample was digested with acid DNAase, aliquots were removed at different times, and digestion was stopped. The reciprocal molecular weights of these samples are given by the lower set of points. Samples were then heat denatured; their reciprocal molecular weights after thermal treatment are given by the upper set of points. See text for further details.

DNA were also degraded by calf thymus–acid DNAase as indicated by acid-soluble oligonucleotide liberation. The similar phage fd DNA was also digested by pure hog spleen–acid DNAase.[3] No kinetic data are avail-

[3] H. E. Schaller, personal communication.

TABLE IV
DEGRADATION OF DENATURED DNA BY ACID-DNAASE PREPARATIONS[a]

DNA Sample	Source	Preparation Method	DNA Concn (μg/ml)	M_w 10^{-6}	R_z	Enzyme	n
(1) B6z-td/1	E	A	121	2.4	590	ET3	2.0
(2) B6z-td/2	E	A	121	2.4	590	ET3	2.0
(3) B3/64	T	A	41.5	2.3	740	ET3	1.6
(4) DL2/27	C		77	2.5	525	ET3	1.4
(5) DL2/28	C		77	2.5	525	ET3	1.8

[a] All degradations were performed in standard acetate buffer. For explanations see footnotes to Table II.

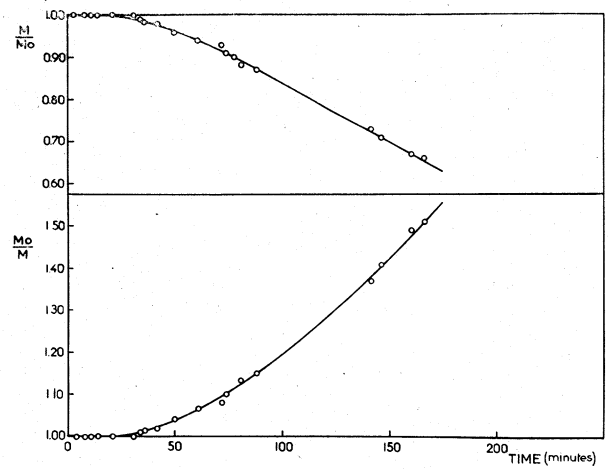

FIG. 7.—Digestion of heat-denatured DNA sample B6-td/1 with acid DNAase (see Table IV).

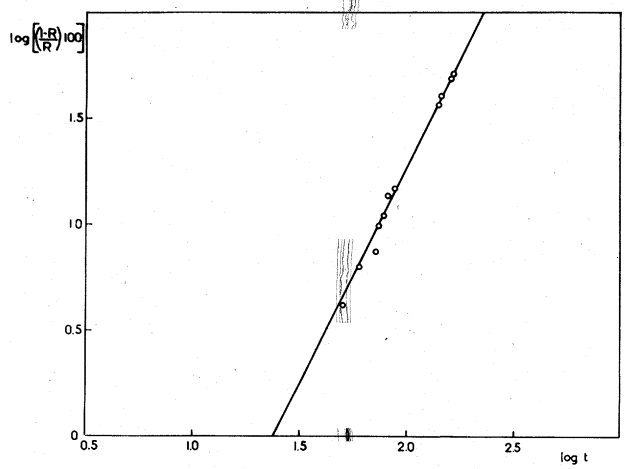

FIG. 8.—Digestion of heat-denatured DNA sample with acid DNAase. Data of Fig. 7 are plotted according to Schumaker *et al.* (1956).

able in these cases because of the scarcity of these DNA preparations.

Heat-Denaturation of Partially Digested DNA.—When DNA samples, partially digested by acid DNAase and ranging in molecular weight from 4 to 1 $\times$ 10^6, were thermally denatured, the percentage molecular-weight decrease caused by heating was found to be larger for the samples of smaller initial molecular weight (Fig. 6; Table III).

Degradation of Heat-denatured DNA by Acid DNAase.—DNA samples, heated for 10 minutes at 100° and fast-cooled showed, upon digestion with acid DNAase, a double-hit kinetic (Figs. 7 and 8; Table IV). The values of n, however, were found to be generally in the range 1.4–1.7. The degradation, as measured by acid-soluble-oligonucleotide liberation, is slower for heat-denatured than for native DNA (Bernardi and Griffé, 1964).

Degradation with Proteolytic Enzymes.—These experiments, as well as those with diisopropylfluorophosphate-treated enzyme, mentioned above, were performed, before acid DNAase had been obtained in a pure form, to rule out the possibility that a proteolytic enzyme was responsible for the single-hit degradation by breaking hypothetical protein "links." They are reported here because they are at variance with results published by Cavalieri *et al.* (1961). Four different samples derived from preparation B6 (from chicken erythrocytes) were used in these experiments: (a) DNA B6 A was obtained by submitting DNA in the saturated NaCl stage (see Materials) to only one chloroform–isoamyl alcohol treatment; (b) DNA B6 B received four more chloroform–isoamyl alcohol treatments in saturated NaCl solution; (c) DNA B6 C was obtained from B6 A by shaking it five more times with chloroform–isoamyl alcohol in Na acetate, μ = 0.15, *p*H 8.6; (d) DNA B6 D was a B6 C sample reaggregated by allowing it to stand 20 days in acetate buffer, *p*H 5.4, μ = 0.15, at room temperature (the solution was saturated with chloroform–isoamyl alcohol).

The results of digestion of the above samples with papain and chymotrypsin are shown in Figure 9. Samples B6 A, B6 B, and B6 D displayed a decrease in molecular weight to final values in the range 4–6 $\times$ 10^6, the lower values being obtained with chymotrypsin. Sample B6 C, which showed at the beginning of digestion the same molecular weight as sample B6 D after papain treatment, did not show any molecular-weight decrease upon incubation with the enzyme.

DISCUSSION

No special comments seem necessary concerning the results obtained with pancreatic DNAase or with proteolytic enzymes. As indicated earlier, all native DNA samples investigated in our laboratory were initially degraded by acid DNAase according to a single-hit kinetics. In spite of all changes introduced into the variable factors involved, it was not possible to find any exception to this rule. The same conclusion was reached when using viscometry instead of light scattering to follow the degradation.[4] These results firmly establish the existence of a single-hit kinetics, a point first suggested on the basis of qualitative evidence by Oth *et al.* (1958).

Using their acid DNAase preparation from mouse sarcoma in order to degrade native calf thymus DNA, Cavalieri and Rosenberg (1961) obtained values of n between 1.5 and 1.7. This result is not necessarily in disagreement with ours, since digestion was performed on sheared DNA samples, where we also find values of n equal to 1.3–1.5.[5]

[4] E. G. Richards, personal communication.
[5] G. Bernardi and C. Sadron, to be published.

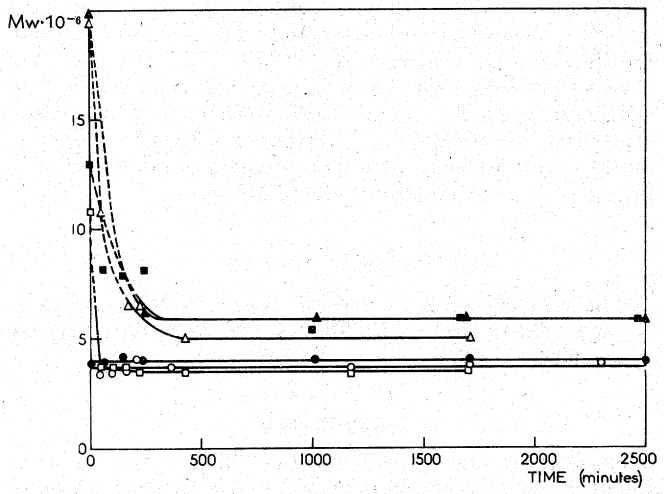

FIG. 9.—Digestion of DNA samples B6 A (triangles), B6 B (squares), and B6 C (circles) with chymotrypsin (open symbols) and papain (solid symbols). DNA concentration was 50 μg/ml for DNA B6 A, 67 μg/ml for DNA B6 B, and 125 μg/ml for DNA B6 C. Molecular weights higher than 6.10^6 are highly doubtful (Froelich *et al.*, 1963). See text for further details.

That the enzyme not only degrades DNA by a single-hit but also by a double-hit mechanism is indicated by the following findings: (a) Titration data obtained by Dr. E. G. Richards in our laboratory,[4] and to be reported elsewhere in detail, show that phosphodiester bond splitting is linear with time down to extremely low molecular weights, a result which justifies the replacement of p with t in equation (2). For a given decrease in molecular weight (down to 0.5×10^6) the number of bonds broken is larger than expected for a pure single-hit kinetics, but much smaller than for a double-hit kinetics. In fact, about ten to twenty breaks (a value likely to be overestimated) were found to be necessary to halve the molecular weight of each parent molecule of $M_w = 6 \times 10^6$, whereas 200 breaks are needed in a double-hit degradation (Thomas, 1956) and about 3 in pure single-hit kinetics in order to obtain the same result.

(b) It has been reported here that $1/M$ increases faster with time after a value of about 1×10^6 has been reached. Since bond splitting is linear with time, this phenomenon is incompatible with the existence of pure single-hit kinetics, and is most easily explained by the superposition of two different mechanisms of degradation. One of them, the single-hit mechanism, is immediately effective in causing a decrease in molecular weight, whereas the second, the double-hit mechanism, also starting immediately after the addition of enzyme, becomes effective only after a time lag.

This conclusion was confirmed by MacHattie *et al.* (1963) using a completely different experimental approach DNA from T5 bacteriophage was digested with acid DNAase and the fragment-size distribution at three time intervals was studied by electron microscopy. If the number of scissions found by MacHattie *et al.* (1963) is plotted against digestion time, a curve is obtained whose shape is very similar to that shown in Figure 5.

(c) The results obtained upon heat-denaturation of partially digested DNA samples (Fig. 6) may be explained by the fact that strand separation, occurring at a high temperature (Doty *et al.*, 1960), liberates more single-stranded fragments from partially digested DNA than from intact DNA because of the presence in partially digested DNA of a certain number of "nicks"

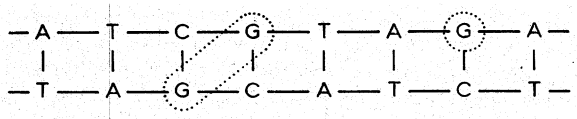

FIG. 10.—Scheme of degradation of DNA by difunctional alkylating agents. From Brookes and Lawley (1961).

introduced by the double-hit action of the enzyme. An interesting finding is the higher degree of residual hyperchromicity (following the rapid cooling of the samples) for the longer-digested samples (Table III). This seems to indicate that the difficulty of re-formation of double-stranded structures upon fast cooling varies inversely with the size of the single-stranded fragments present in the solution at high temperature. These results are similar to those reported by Geiduschek (1962) for his type I reversibility. A dependence of renaturation (type II reversibility of Geiduschek) upon DNA molecular weight was also reported by Marmur and Doty (1961).

The data presented in Figure 6 permit one to estimate the ratio of total bonds broken to bonds broken by the single-hit kinetics as equal to about 3, which means two breaks by the double-hit kinetics per one break by the single-hit kinetics. However, since this calculation requires several unwarranted assumptions such as complete strand separation upon heating, no interchain association upon cooling, and no DNA hydrolysis, the foregoing result may be considered only as being in rough agreement with titration data.

(d) The degradation of DNA from phages ϕ X 174 and fd indicates that the enzyme is also able to hydrolyze single-stranded molecules. (e) Heat-denatured DNA samples are digested according to double-hit kinetics. This apparently puzzling result is not so surprising if one considers that heat-denatured DNA regains a very largely double-stranded structure upon cooling. A certain number of single-hit attacks seem to be present as suggested by generally low values found for n. This point will be considered in more detail elsewhere.[5]

While the double-hit mechanism is essentially identical with that exhibited by pancreatic DNAase and does not need any special comment, several hypotheses may be put forward to explain the single-hit mechanism of degradation: (a) DNA has some extent of strand separation at the acid pH used in the enzymatic digestion. This explanation (Oth *et al.*, 1958) certainly is incorrect, because no difference in the intrinsic viscosity or in the radius of gyration can be found for the same DNA solution at pH 7 or 5 ($\mu = 0.15$).[5] Furthermore the same kinetics is found at pH 7.0, $\mu = 0.005$, as at pH 5.0, $\mu = 0.15$.

(b) DNA has some interruptions on one or the other strand, which may be primary or secondary to enzymatic attack during the extraction procedure. The single-hit degradation would then correspond to the breakage of bonds opposite the interruptions (these bonds also should not be susceptible to pancreatic DNAase; otherwise the degradation by this enzyme would give a single-hit kinetics). This hypothesis also appears to be incorrect, as judged from the following evidence. First, it is unlikely that all the DNA samples used in this work had undergone, roughly at the same extent, an enzymatic degradation during the preparation. Second, when random breaks were purposely introduced by mild enzymic digestion, their existence became evident upon heat denaturation (Table III), whereas no intact DNA sample used in this work showed more than a 40% decrease in molecular weight upon thermal treatment.

(c) DNA is made up of two continuous filaments, and both are broken at the same level (Oth *et al.*, 1958). Two cases may be considered, depending whether the two breaks are simultaneous or successive. The case of two independent successive breaks is excluded because, unless the two breaks follow each other extremely closely in time, this would show up as a double-hit kinetics. The second hypothesis of a simultaneous breakage of both DNA strands at the same level appears to be the correct one.

In relationship with a possible visualization of the process involved in the single-hit action, it may be interesting to consider a chemical degradation of DNA, which is formally identical to that provoked by acid DNAase. This is the degradation of DNA by difunctional alkylating agents, as studied by Brookes and Lawley (1961). Under their experimental conditions, alkylation has been shown to occur at N_7 of guanine, monofunctional agents yielding 7-alkylguanines, and difunctional agents yielding, in addition, di(guaninyl) derivatives. This latter event has been shown to occur only when two guanines are found on opposite strands, as in Figure 10. Alkylated DNA decomposes with loss of the alkylated guanines and subsequently the corresponding phosphodiester bonds are hydrolyzed. Therefore the net result is that alkylation by monofunctional agents gives rise to a random degradation of DNA of the type obtainable with pancreatic DNAase, whereas alkylation by difunctional agents causes a degradation which involves both single-hit and double-hit kinetics, therefore simulating the action of acid DNAase.[6]

These data led Bernardi and Sadron (1964) to put forward the hypothesis that acid DNAase might be a protein with two active sites. The enzyme would then be able to split both DNA strands simultaneously at sites where susceptible nucleotide sequences exist at the same level on opposite strands, or to break only one strand when a susceptible site exists on one chain only. The almost complete disappearance of the single-hit kinetics in heat-denatured DNA might indicate that the re-formation of sites susceptible to double breaks (by rematching of the sequences on the opposite strands) had not occurred to a significant extent upon fast cooling. Another possibility is that the double-stranded structure of fast-cooled heat-denatured DNA is sufficiently different from that of native DNA to make the single-hit attack impossible. This latter interpretation deserves some consideration in view of the finding that in the recently discovered competitive inhibition of acid DNAase by polyribonucleotides[7] double stranded poly-A or poly-C do not exert any action whereas soluble RNA or poly-A–poly-U are very powerful inhibitors, a phenomenon possibly related to the structural differences existing among the polyribonucleotides mentioned (see Tomita and Rich, 1964, for example).

Alternatively, one might think that the single-hit degradation is due to the splitting of special bonds like the phosphoserine-nucleoside linkages postulated by Bendich and Rosenkranz (1963), for example. In connection with this second hypothesis it may be interesting to recall that acid DNAase also has a phosphodiesterase activity on Ca[bis-(*p*-nitrophenyl)phosphate]$_2$ and on the *p*-nitrophenyl esters of nucleoside-3′-phosphates (Bernardi and Griffé, 1964).

[6] It should be pointed out that this description of the DNA degradation by alkylating agents gives a rather idealized picture of the process involved; in fact, for example, the double-hit kinetics expected with monofunctional agents was not found experimentally (J. A. V. Butler, personal communication).

[7] G. Bernardi, to be published

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